Supporting Information

Ding et al. 10.1073/pnas.1003919107

SI Materials and Methods

Morpholino-3′-NH_2 AONs, AGC-TCA-AAG-CCA-TTT-CTC-CGC-TGA-C to laminin-411 α4 chain, CTA-GCA-ACT-GGA-GAA-GCC-CCA-TGC-C to laminin-411 β1 chain (1), and the same sequences with a Lissamine end modification were custom-made by Gene Tools (Philomath, OR). Rat anti-mouse TfR mAb R17217 (Ms) and mouse anti-human TfR mAb RVS-10 (Hu) were from Southern Biotech. Highly purified, endotoxin-free poly (β-L-malic) acid, Mw (weight-averaged) = 100 kDa, polydispersity = 1.1, was obtained from the culture broth of *P. polycephalum* (2), after removal of endotoxin as described (3). $mPEG_{5000}$-amine and maleimide-PEG_{3400}-maleimide were from Laysan. NPD-PC and egg yolk phosphatidylcholine were from Avanti Polar Lipids, Rhodamine-maleimide was from Invitrogen. NH_2-Leu-OEt and NH_2-Leu-Leu-Leu-OH were from Bachem Americas Inc. Calcein and cholesterol were from Sigma-Aldrich.

Syntheses. PMLA conjugates with H_2N-Leu-OEt (LOEt), H_2N-Leu-Leu-Leu-OH (LLL), H_2N-Leu-Leu-Leu-NH_2 (LLL-NH_2) were synthesized as follows: to 1 mL solution of 73 mg PMLA (0.63 mmol equivalent of malic acid) in acetone, a mixture of NHS and dicyclohexylcarbodiimide in 2 mL dimethyl formamide (DMF) was added. After 4 h stirring at room temperature (RT), dicyclohexylurea was filtered and the volume reduced to 0.5 mL by evaporation. The mixture with 2 mL pyridine and leucine peptide (0.25 mmol equivalent to 40% malyl groups) dissolved in 0.5 mL DMF/24 μL trifluoracetic acid was kept at RT for 2 h. The reaction was completed after 30 min with the addition of 20 μL triethylamine, verified by TLC/ninhydrin test. Unreacted NHS-esters were hydrolyzed by the addition of water. Conjugates were purified over PD-10 columns (GE Healthcare). Nanoconjugates containing AONs and/or antibodies were synthesized from the 2-mercapto-1-ethylamine containing preconjugates and the mixtures of AONs or mAbs as described (4). Fluorescent dyes like Alexa Fluor 680 or rhodamine were conjugated as their maleimide derivatives to the platform pendant sulfhydryls. Synthesized nanoconjugates were lyophilized and stored at −80 °C; non lyophilized nanoconjugates were stored at 4 °C for no longer than 2 d. PBS solutions for in vivo treatments were sterilized by membrane filtration.

To achieve stoichiometrically defined conjugation, reactions were conducted with limiting amounts of reagents to completion, controlled by TLC/ninhydrin staining. Contents of NHS, sulfhydryls, and oligonucleotides were quantified by amine consumption, Ellman color reaction, and/or quantitative HPLC after disulfide cleavage with dithioerythritol or glutathione, respectively (4). Amino acids were quantified by RP- HPLC after hydrolysis of conjugates in 6 M HCl at 100 °C and colorimetry with trinitrofluorobenzene following standard protocols (4).

Purity tests included TLC/ninhydrin staining and size exclusion high-performance liquid chromatography (sec-HPLC, BioSep-SEC-S-3000 column; Phenomenex) at 220, 260, and 280 nm wavelengths (Elite LaChrom system; Hitachi). Specific antigen recognition and tandem configuration of mAbs were tested by ELISA (5). Acquisition of particle sizes and ζ potentials (pH, 7.4; 1–10 mg/mL samples) at 20 °C in the presence of PBS or 10 mM NaCl, respectively, was performed with Zetasizer Nano System ZS90 (Malvern Instruments) using their standard protocols. The pH dependence of ζ potential was assessed with 200 μg/mL either P/LLL or P/LOEt alone and in the absence/presence of 160 μM lipid (as liposomes). Weight averaged molecular weights were measured by sec-HPLC using polystyrene sulfonates as standards. Absolute molecular weights and second virial coefficients (Rayleigh light scatter equation) of nanoconjugates were measured with Zetasizer using experimentally obtained refractive index increments (6).

Liposome Leakage Assay. A fluorescent assay for calcein release from loaded phosphatidylcholine/cholesterol liposomes was used to determine the membrane disruption activity of various polymer conjugates. Liposomes were prepared by the extrusion method (7). Briefly, the mixture of egg phosphatidylcholine and cholesterol (molar ratio, 2:1) dissolved in $CHCl_3$/MeOH (vol/vol, 2:1) was dried under a stream of nitrogen. The lipid mixture was hydrated with 5 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, and 90 mM calcein, followed by 19 extrusions through 0.1-μm polycarbonate membrane using miniextruder (Avanti Polar Lipids). To assess leakage at different pH values, nanoconjugates were serially diluted in 95 μL of buffers of different pH in a plate, 137 mM Hepes buffer pH 7.4, and 137 mM citrate buffer pH 5.0. Triplicates were mixed with 5 μL liposome suspensions in 5 mM hepes buffer, 150 mM NaCl pH 7.4 (final lipid concentration 160 μM). Complete leakage of calcein was achieved with the addition of 0.25% (vol/vol) Triton-X 100 solution of respective buffers. After 1 h at room temperature, fluorescence was read by an ELISA reader at 485-nm excitation wavelength and 535-nm emission wavelength. Triton-X 100, 0.25% (vol/vol), was used as a reference for 100% leakage.

Giant liposomes were prepared by the evaporation method (8). Briefly, the mixture of egg phosphatidylcholine and cholesterol (molar ratio, 2:1) containing 1-oleoyl-2-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino] lauroyl]-*sn*-glycero-3-phosphocholine (NBD-PC) (1% relative to egg phosphatidylcholine) dissolved in $CHCl_3$ (lipid concentration 0.1 M) and 20 μL of this solution was to added to a 100-mL, round-bottom flask containing 980 μL of chloroform and 100 μL of methanol. Seven milliliters of Hepes buffer (10 mM, pH 7.4) was added to the lipid solution and the organic solvent was removed by rotary evaporation under reduced pressure at 40 °C at 45 rpm. The giant liposomes have the same lipid composition as the liposomes for the leakage assay except prepared with additional 1% (molar) green fluorescent NBD-PC. For confocal microscopy, the liposomes were incubated with 20 μg/mL of P/LLL and P/LOEt covalently labeled with 1% rhodamine in Hepes buffer (10 mM, pH 7.4) for 30 min at room temperature.

Cell and Tissue Section Immunostaining and Colocalization Studies. For in vitro colocalization studies, U87MG cells were seeded at 1×10^5 on Lab-Tek chamber slides (Thermo Fisher Scientific) for 24 h. The cells were washed once with serum-free medium followed by incubation in 500 μL serum–free medium with fluorescently labeled P/LLL/AON/Hu conjugate at a concentration of 30 μg/mL antibody. The polymer was tagged with Alexa Fluor 680 (maximum excitation/emission wavelength: 679/702 nm; Invitrogen), and AONs were conjugated with disulfide Lissamine (575/593 nm). Endosomal membranes were stained with 5 μg/mL of the lipophilic styryl red fluorescent dye FM 1–43 (479/598 nm; Invitrogen). After 30 min incubation, cells were washed three times in serum free media and finally incubated in fresh media with serum.

A TCS SP5× spectral scanner (Leica Microsystems) was used for confocal microscopy. Image stacks of 246 × 246 μm in size and 7.5 μm in depth of live U87MG glioma cells were acquired with a HCX PL APO CS 63.0 × 1.20 lens. Live cells were cultured on chamber slides. A temperature of 37 °C was maintained

by a separate lens and chamber heating system. Live confocal images were taken 0 h and 3 h after labeled drug removal. The spectral settings were optimized for fluorescence intensity of each fluorophore excluding bleed-through, i.e., for FM 1–43, excitation 475 nm and emission 525–585 nm; for Lissamine, excitation 576 nm and emission 585–632 nm; for Alexa Fluor 680, excitation 670 nm and emission 685–750 nm.

The extent of colocalization between stained membrane, polymer backbone, and morpholino AON was estimated by calculation of Pearson's correlation coefficient [R(r)] using ImageJ "Co-localization Finder Plugin" (http://rsbweb.nih.gov/ij/) (9) with raw image. Colocalization was determined at 0 h and 3 h after drug removal.

The colocalization of AON and PMLA in vivo was further assessed on brain tumor sections after additional immunostaining for vWF to reveal blood vessels. Cryostat sections were fixed in acetone at −20 °C for 20 min, and then incubated with rabbit polyclonal anti-vWF antibodies (1:100; Millipore) at RT for 1 h. The signals were detected with FITC-conjugated secondary antibodies (1:200; Millipore). The sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories), coverslipped, and examined under the confocal microscope.

The same procedure was used to reveal the immunostaining patterns of laminin-411 α4 and β1 chains on tumor sections. Primary antibodies were rabbit polyclonal anti-α4 chain LG1-3 domain (1,100+, 1:200) (10) and rat monoclonal anti-β1 chain (1:20, clone LT3; Abcam). Both antibodies stained all vessels on sections as confirmed by colocalization with vWF. For each laminin chain, all sections were photographed with the same exposure time. Morphometric analysis of vessel density was performed with ImageJ 1.43c software. Images of five random nonoverlapping fields of view (field area = 0.245 μm^2) per tumor ($n = 5$ in each group; 25 fields per group) were obtained with 20× Plan Apochromat objective in an Olympus BX40 microscope (Olympus) equipped with MicroFire color digital camera (Optronics). Because of colocalization of α4 and β1 laminin chains (Fig. 5*D*) and with vWF, only grayscale pictures of β1 laminin chain were analyzed. The pictures were converted to binary files with threshold only showing the microvessels, followed by measuring the percentage of vessel area (11) per 20× field. Data were compared between PBS, P/LOEt/AON/Hu/Ms, and P/LLL/AON/Hu/Ms treatment groups using ANOVA with Newman–Keuls multiple comparison test.

Western Blot Analysis. Glioblastoma U87MG or T98G cells (1×10^6) were suspended in 10-cm dishes in serum-containing MEM medium on day 0. After replacement with serum-free MEM medium with Galardin/proteinase inhibitor mixture on day 1, cells were treated with conjugates containing 1.4 μM AON and with controls including PBS, free morpholino AON, or a conjugate not containing LLL. On day 2, treatment was repeated with fresh medium and reagents. On day 5, culture supernatants were collected and 10-fold concentrated by centrifugation over Centriplus-100 (Millipore). For Western blotting, samples of 20 μg protein were separated by reducing SDS/PAGE using 8% Tris-glycine gels (Invitrogen). After blotting onto nitrocellulose membrane (Invitrogen), proteins were identified by specific antibodies and chemiluminescent detection provided by Immune-Star kit using alkaline phosphatase-conjugated anti-mouse GAM-AP (1:3,000; Bio-Rad) or anti-rat IgG-AP (1:10,000; Jackson ImmunoResearch). Primary mAbs for protein detection were mouse anti-laminin α4 (1:1,000, clone 8F12; a gift from Dr. Sekiguchi, Department of Biological Sciences, Osaka University, Osaka, Japan), rat anti-laminin β1 (1:1,000, clone LT3; Abcam), and mouse anti-human fibronectin eighth type III repeat (1:1,000, clone 568) (1).

1. Khazenzon NM, et al. (2003) Antisense inhibition of laminin-8 expression reduces invasion of human gliomas in vitro. *Mol Cancer Ther* 2:985–994.
2. Lee BS, Holler E (2000) Beta-poly(L-malate) production by non-growing microplasmodia of Physarum polycephalum. Effects of metabolic intermediates and inhibitors. *FEMS Microbiol Lett* 193:69–74.
3. Aida Y, Pabst MJ (1990) Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J Immunol Methods* 132:191–195.
4. Lee BS, et al. (2006) Polycefin, a new prototype of a multifunctional nanoconjugate based on poly(β-L-malic acid) for drug delivery. *Bioconjug Chem* 17:317–326.
5. Fujita M, et al. (2007) Brain tumor tandem targeting using a combination of monoclonal antibodies attached to biopoly(β-L-malic acid). *J Control Release* 122: 356–363.
6. Hiemenz PC (1984) *Light Scattering by Polymer Solutions in Polymer Chemistry: The Basic Concepts* (Marcel Decker Inc, New York).
7. Van Rossenberg SM, et al. (2002) Targeted lysosome disruptive elements for improvement of parenchymal liver cell-specific gene delivery. *J Biol Chem* 277: 45803–45810.
8. Moscho A, Orwar O, Chiu DT, Modi BP, Zare RN (1996) Rapid preparation of giant unilamellar vesicles. *Proc Natl Acad Sci USA* 93:11443–11447.
9. Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics International* 11:36–42.
10. Talts JF, et al. (2000) Structural and functional analysis of the recombinant G domain of the laminin α4 chain and its proteolytic processing in tissues. *J Biol Chem* 275: 35192–35199.
11. Warin R, Xiao D, Arlotti JA, Bommareddy A, Singh SV (2010) Inhibition of human breast cancer xenograft growth by cruciferous vegetable constituent benzyl isothiocyanate. *Mol Carcinog* 49:500–507.

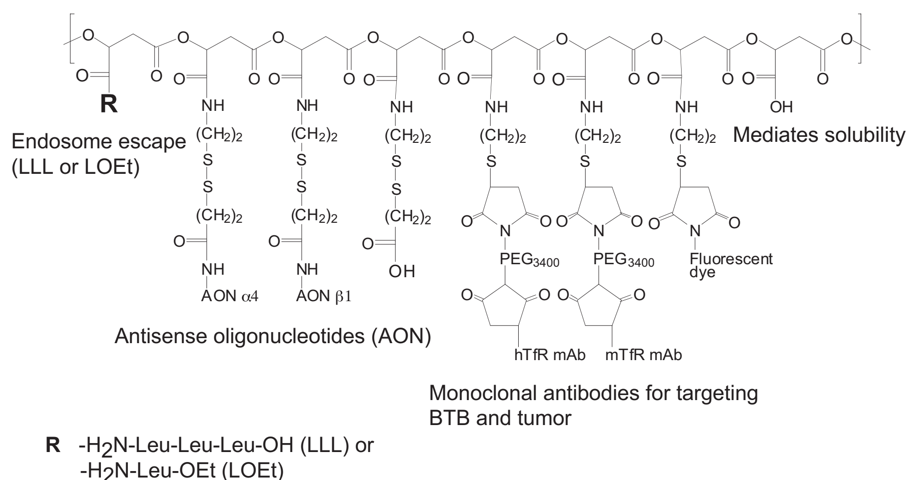

Fig. S1. Schematic formula of the nanoconjugate containing a full set of conjugated functional groups.

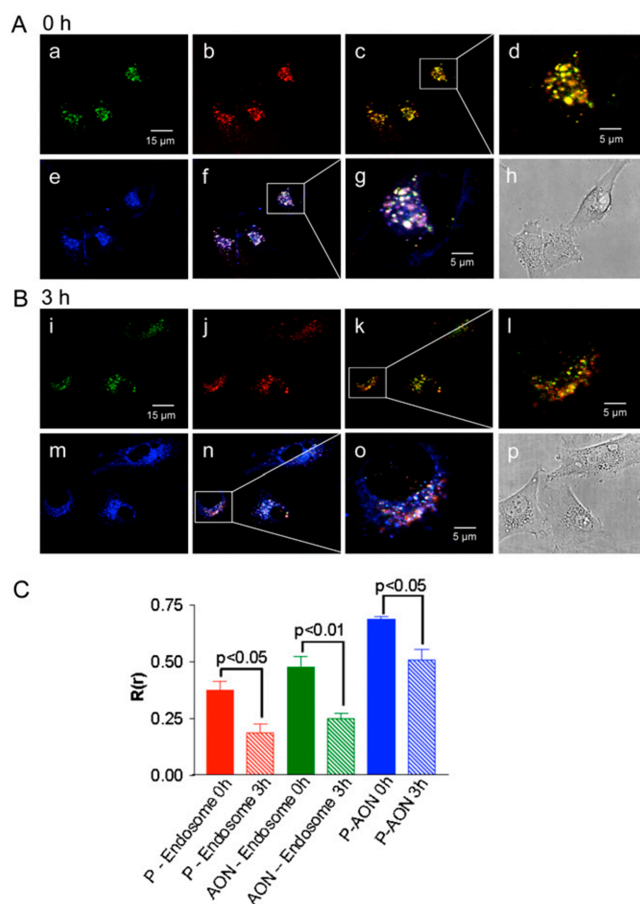

Fig. S2. Confocal in vitro localization of P/LLL/AON/Hu after uptake by U87MG cells following double labeling of the polymer PMLA platform with Alexa Fluor 680 and of AON with Lissamine. U87MG cells and nanoconjugate P/LLL/AON/Hu were incubated for 30 min at 37 °C. Representative fluorescence pictures were then taken at 0 h (*A*) and 3 h (*B*) after the incubation. (*A*, *a*, and *B*, *i*) Polymer platform labeled with Alexa Fluor 680 (green). (*A*, *b*, and B, *j*) AONs labeled with Lissamine (red). (*c* and *k*) Merge of polymer platform panel (A, *a*, and B, *i*) and AONs panel (*A*, *b*, and B, *j*), respectively; colocalization is indicated by yellow staining, which predominates. (*A*, *d*, and *B*, *l*) close-up of areas marked in *A*, *c*, and *B*, *k*, respectively, allowing to better appreciate colocalization (yellow). (*A*, e and *B*, *m*) Endosomes stained with FM1-43 (blue). (*A*, *f*, and *B*, *n*) Respective merges of panels for polymer platform (*A*, *a*, and *B*, *i*), AONs (*A*, *b*, and *B*, *j*), and endosomes (*A*, *e*, and *B*, *m*); colocalization is indicated by white staining. (*A*, *g*, and *B*, *o*) Close-up of areas marked in *A*, *f*, and *B*, *n*, respectively, allowing to better appreciate colocalization (white). (*A*, *h*, and *B*, *p*) Phase contrast of respective cells. (*C*) Pearson's correlation coefficients R(r) for the colocalization of platform-membranes, AONs-membranes and platform-AONs were calculated for 0 h and 3 h. Mean values ± SEM refer to seven pictures containing 10 cells each. Note substantial colocalization of polymer platform with AON and of both of them with endosomes at 0 h. This colocalization significantly decreased at 3 h, consistent with endosome rupture and drug release into the cytoplasm.